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PRINCIPAL INVESTIGATOR: David H. Walker, M.D.
Juan P. Olano, M.D.

CONTRACTING ORGANIZATION: University of Texas Medical School
Galveston, Texas 77555-0657

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David H. Walker, M.D.
Juan P. Olano, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Texas Medical School
Galveston, Texas 77555-0657

E-Mail: dhwalker@utmb.edu

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13. ABSTRACT (Maximum 200 Words)

The purpose of this study is to utilize adaptein libraries coded within pantropic retroviral vectors that confer protection against rickettsial pathogens and to study the molecular pathogenesis of rickettsioses. The following specific aims were proposed: 1) To establish heterogeneous cell populations, with each cell expressing a unique member of a complex combinatorial peptide-based (e.g., adaptein) library and challenge with *R. prowazekii*, *R. rickettsii*, and *O. tsutsugamushi*; 2) To determine the role of NF-κB, cytokines, ROS and NO in intracellular killing of rickettsia-infected monolayers containing adapteins and 3) To characterize signal transduction pathways modulating the cytoskeletal events responsible for the increased vascular permeability. During the third year of the project, rickettsial challenges performed with the transfected rat derived microvascular endothelial cells and the human brain primary microvascular endothelial cells were somewhat disappointing. Expansion of the "resistant colonies" was not possible. Two other human microvascular endothelial cell lines were acquired (cerebral and dermal). These cell lines are far more susceptible to rickettsial infection than their predecesors. We have transfected successfully both cell lines with the adaptein-containing retroviral vectors and "resistant colonies" were obtained after three consecutive challenges. However, expansion of the colonies continues to be elusive. Supernantants of infected monolayers were shown to affect the endothelial monolayer permeability dramatically, suggesting the presence of soluble factors, yet to be characterized, responsible for such changes. Excellent progress continues to be made on the development of an in vitro model for the study of microvascular permeability using human derived microvascular cells of cerebral and dermal origin.

14. SUBJECT TERMS
Rickettsiae, orientia, adapteins, scrub and epidemic typhus

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I. INTRODUCTION

Rickettsiae are obligately intracellular organisms that have evolved in close association with an arthropod host. Diseases caused by these organisms are still prevalent in many parts of the world and include Rocky Mountain spotted fever (the most common rickettsiosis in the US), epidemic and endemic typhus (1-4). The latter two are still responsible for thousands of deaths around the world every year. Rickettsial diseases are found in every continent except for Antarctica and are considered both emerging and re-emerging infectious diseases. In addition, *Rickettsia prowazekii* and *R. rickettsii* are listed in the Select Agents Act and are part of the Centers for Disease Control and Prevention (CDC) and NIH category B agents and the North Atlantic Treaty Organization (NATO) select agent list for their potential use as bioterrorist/biowarfare agents (5-8). The most feared complications of rickettsial infections are the development of severe cerebral and pulmonary edema leading to permanent neurologic sequelae or death owing to respiratory failure (3,4). The target cell of rickettsial pathogens is the endothelium lining the vessels of the microvasculature, as demonstrated by studies performed on autopsy cases dying of rickettsioses and animal models (9,10). The purpose of this study is to utilize adaptein libraries coded within pantropic retroviral vectors that confer protection against rickettsial pathogens. In addition, molecular pathogenesis of rickettsioses is being studied by developing *in vitro* models to study endothelial permeability and intracellular rickettsial killing in both wild type and adaptein protected cells. The long term objective of this proposal is to develop new treatments for rickettsioses and to identify novel molecular targets of rickettsial pathogenesis that would provide sites for new therapeutic interventions, and to eventually use these targets to develop effective and rapid BWT countermeasures. The new therapeutic interventions are justified due to the narrow range of antimicrobial agents available for rickettsiae and *Orientia,* emergence of chloramphenicol- and tetracycline-resistant strains of *Orientia,* and the possibility of genetically engineered resistance.

I. RESEARCH PROGRESS

During the third year of funding for this project, we have been able to continue the experiments related to all specific aims.

A. Specific aim # 1: To utilize retrovirally encoded adapteins to generate cell monolayers resistant to rickettsial challenge.

During the third year 6-mer, 12-mer, and 18-mer adapteins with the EGFP scaffold were used for the challenging experiments. As described in our second progress report, these experiments were done with rat-derived brain microvascular endothelial cells (RBE4). The major difficulty that we faced last year was the expansion of the "resistant colonies" containing 20-25 cells. Several methods were used to try to expand these colonies including enrichment of the medium, addition of non-transformed RBE4 cells in order to increase the "quorum sensing" of the monolayer, and use of small culture vessels in order to decrease the area of growth and therefore increase "quorum sensing". Unfortunately, none of these modifications was able to increase the expansion of these colonies.

During the third year we also optimize the protocols to transfect two new human microvascular endothelial cells lines, namely SV-HCEC and TIME (SV40-transformed human microvascular endothelial cells and telomerase immortalized microvascular endothelial cells, respectively), both of which are more susceptible to rickettsial infection and cell death as shown by experiments performed with Electronic Cell Substrate Impedance Sensing (ECIS; see specific aim 3). Both cell monolayers were infected with the pantropic retroviral vectors containing 6-, 12-, and 18-mer adapteins and challenged as described in previous progress reports with 25 MOI of *R. rickettsii, R prowazekii* and *O. tsutsugamushi.* After 96 hours, attached surviving cells which consisted of both EGFP-positive and negative cells, were trypsinized, replated, and allowed to recover and grow to confluence in T-25 flasks before being rechallenged with 15 MOI of each pathogen, respectively. After the second round of challenge, 10-15 foci of EGFP-positive cells were observed. These "resistant colonies" were gently trypsinized, replated onto wells of a 12-well plate and allowed to recover for 3

days. A third challenge was performed using the same inoculum and 10-20 EGFP-positive resistant colonies were observed in each flask. Attempts to expand these colonies have been unsuccessful.

With the recent acquisition of a flow cytometer/cell sorter (FACSCalibur, Beckton-Dickinson) for use in our BSL-3, we will be able to use a different protocol to try to sort the cells resistant to the rickettsial challenge in cell suspensions. We will use both endothelial cell monolayers that will be gently trypsinized and macrophage cell lines susceptible to rickettsial challenge such THP-1 cells (a human monocytic leukemia cell line whose maturation to macrophage phenotype can be induced in vitro by using dexamethasone) that grow in suspension and therefore will be easy to sort out. Macrophages, indeed are a secondary target for several rickettsial organisms.

B. Specific aims #2 and #3: To determine the roles of NF-κB, cytokines, ROS and NO in intracellular killing of rickettsia-resistant monolayers. To characterize signal transduction pathways modulating the cytoskeletal events responsible for the increased vascular permeability seen in rickettsial infections.

Experiments during the second year were performed with a rat derived microvascular endothelial cell and a primary human brain microvascular endothelial cell.

During the third year we decided to evaluate the recently acquired TIME and SV-HCEC cells. They were seeded on disposable arrays with gold electrodes at a density of 10^5 cells. Resistance across the cell monolayer (transendothelial resistance or TER) was monitored with Electronic Cell-substrate Impendance Sensing (ECIS, Applied Biophysics, NY) until TER stabilization occurred. The TIME monolayers were then infected with different MOI of *R. rickettsii*, ranging from 5 to 100 and SV-HCEC cells were infected with MOI ranging from 10-50. For SV-HCEC monolayers, permeability increases were observed soon after infection of the monolayers and reached 30% after 3 hours. By 6 hours, differences between the different MOI became apparent and monolayers infected with 50 MOI revealed a 50% permeability increase, whereas monolayers infected with 10 MOI stabilized at 30-35% increase. Subsequent experiments were performed with 15 MOI since

with this amount of inoculum, the cells in the monolayer would not die precipitously and would allow us to study the early events in the monolayer leading to permeability changes. At 15 MOI, SV-HCEC monolayers showed an initial drop of 20-25% after 6 hours which remained stable until 32 hours post-infection after which a more pronounced drop was documented, reaching 70-80% in 48 hours (Fig 1). Cell death rates were also studied during the experiment by using propidium iodide. At 24 hours, no statistically significant differences ($p>0.05$) were noted in cell death rates between infected and non-infected monolayers, suggesting that changes in permeability during the first 24 hours were due to infection per-se and not to cell death. However, at 48 hours the differences were statistically significant ($p=0.0008$) regarding cell death between infected and non-infected monolayers. This finding explains the precipitous increase in permeability after 34 hours of infection previously described (Fig 2).

In order to asses the effect of cytokines (IL-1β, IFN-γ, and TNF-α) on rickettsia-infected SV-HCEC monolayers, we used 15 MOI of *R. rickettsii* and evaluated permeability for 24 hours. After 24 hours of infection, each of the three cytokines and the different combinations were added at concentrations of 5 and 20 ng/ml. During the first 24 hours post-infection, permeability in the monolayers increased by 20% and remained so until cytokines were added at 24 hours after which a further 10-15% increase in permeability was observed after addition of IL-1β and a 15-20% increase after addition of TNF-α (Fig 3-4). No major differences were noted between 5 and 20 ng/ml of the cytokine. A dramatic increase was then observed between 15 and 19 hours (up to 80%) after addition of both cytokines. This dramatic increase was due to cell death as evidenced by loss of micromotion in resistance tracings. In addition, the monolayers exposed to the cytokines showed marked increase in permeability at least 2 hours earlier than the infected monolayers without cytokines. Addition of IFN-γ increased permeability in infected monolayers by 5-10% for 15 hours after being added to the culture medium and a dramatic increase (up to 80%) similar to the one caused by TNF-α and IL-1β was observed between 17 and 19 hours (Fig 5). The combination of TNF-α and IL-1β did not increase permeability in the monolayer as compared to the monolayers exposed to either cytokine alone (Fig 6). Addition of IFN-γ and

TNF-α increased permeability to the same level as the one observed with TNF-α alone (Fig 7). A similar result was observed when IFN-γ and IL-1β were used in combination (Fig 8). The addition of all three cytokines increased permeability by 20% when compared to infected monolayers without cytokine treatment, a similar increase as the one observed for the combination of TNF-α and IL-1β (Fig 9). These results suggest that TNF-α and IL-1β have the greatest effects on permeability of the monolayers. The results also suggest that the monolayers have a certain range of response that plateaus at a certain level of cytokines in the medium and does not progress any further in the presence of more cytokines.

In order to evaluate the effects of reactive oxygen species (ROS) on the monolayer permeability, 10 U/ml of catalase, 1 mM of N-acetyl-cysteine, and α-tocopherol at a concentration of 30 μM were added to rickettsia-infected monolayers. All three compounds were added both before, and at the time of infection of the monolayers. No differences were observed between infected or non-infected monolayers when catalase or N-acetyl-cysteine were added to the medium. However, pre-treatment of the monolayers with α-tocopherol prevented a 5-10% increase in permeability as compared to infected monolayers that were not pre-treated with α-tocopherol. Cell survival in the monolayer was marginally improved as shown by loss of micromotion in the non-treated monolayers 2 hours earlier when compared to the treated monolayers (Fig 10-12).

We have observed several interesting responses to rickettsiae that most-likely demonstrate a rickettsiae-dependent response resulting in increased microvascular permeability. Specifically we have seen endogenous nitric oxide production in these cells in response to infection with *Rickettsia rickettsii* (MOI 10). Cell culture supernatants contained higher detectable levels of nitrite, a by-product of nitric oxide production, in rickettsiae-infected cells than in mock-infected cells (Fig 13). At this time we are unsure whether the levels of NO produced by these cells, most likely by the endogenous eNOS enzyme, is sufficient to cause any nitric oxide-dependent changes in permeability. However, we were able to show a rickettsiae-dependent response of SV-HCEC to cell culture supernatants of infected cells. More

specifically, supernatant from rickettsiae-infected cells were filter sterilized and mixed 1:1 with fresh cell culture media. This was fed to confluent SV-HCEC grown on 8W10E ECIS electrodes and the transendothelial electrical resistance was monitored by taking a resistance measurement every 2 minutes. Supernatant from cells infected for 24 hours caused a transient decrease in electrical resistance with levels returning to near normal within 48-72 hours. Likewise, supernatants from cells infected for 48 hours caused a terminal decrease in resistance resulting in monolayer detachment within 48-72 hours (Fig 14). Experiments are currently underway to expand on these results and determine the origin of this response, including detailed analysis of cytokines released into the supernatant by Luminex technology.

The role of calcium in endothelial permeability was studied by using verapamil (a calcium channel blocker), BAPTA (an extracellular calcium chelator) and dantrolene (an inhibitor of intracellular calcium release from the endoplasmic reticulum). Each of the calcium blockers was added to monolayers 1 hour before infection after which the medium was replaced with either normal medium and medium containing the calcium blocker. Pre-treatment of the monolayers with verapamil followed by infection and discontinuation of the calcium blocker did not reveal major differences when compared to rickettsia-infected monolayers without calcium blockers. Infection of the monolayers followed by continued use of verapamil reveal a 5-10% increase in permeability across the monolayer, suggesting that blocking entrance of calcium from the extracellular fluid might interfere with normal homeostasis of junctional proteins (Fig 15-16). This observation was further confirmed by continuous addition of dantrolene to the medium, which induced a further 30-35% increase in permeability at 30 hours post-infection when compared to rickettsia-infected monolayers without dantrolene (Fig 15-16). Pre-treatment of the monolayers with dantrolene for 1 hour followed by replacement of medium without dantrolene after infection of the monolayer also increased permeability by up to 5% when compared to rickettsia-infected monolayers (Fig 15-16)

Repeated attempts to demonstrate occluding and claudin expression in all cell lines that we have used to date have been unsuccessful. We have been able to demonstrate ZO-1 expression in SV-HCEC cells. However, recent reports

have found that expression of ZO-1 does not imply tight junction formation as ZO-1 seems to be part of adherens junction proteins complexes, as well. Therefore, our results apply to modifications of adherens junctions leading to increased permeability across the monolayers. In order to study changes in tight junctions associated with rickettsial infections, we decided to use primary microvascular endothelial cells derived from mouse brains. Therefore we sought to develop a method to efficiently isolate and culture microvascular endothelial cells from mouse brains. Briefly, brains from male C3H/HeN mice were aseptically removed homogenized in a glass dounce homogenizer. The brain homogenates were centrifuged in 15% dextran to isolate the microvessels from the foamy myelin containing cells. The resulting pellet was digested in 1mg/ml collagenase/dispase for 1 hour at 37°C. The digested microvessels were then selected for using CD31-coated Dynabeads and were cultured on collagen-coated tissue culture flasks. The microvessels were cultured in DMEM/F12 + 10% plasma-derived serum + 100ug/ml heparin + 100 ug/ml ECGS. These cells were positive for the uptake of Ac-LDL, a marker for microvascular endothelium, and expressed the cobblestone morphology typical of microvascular endothelial cells (Fig17). We were able to confirm that these cells expressed the proteins occludin and ZO-1 in association with the intracellular borders indicating the presence of intact tight junctions (Figure 18). We feel that these cells will allow us to accurately investigate the changes that occur in rickettsiae-infected microvascular endothelial cells that may directly affect endothelial barrier function as it relates to tight junction proteins. Because this grant covers only in vitro experiments and there is not animal protocol approved with this project, brains were harvested from mice used for other experiments in our laboratory and covered by an approved animal protocol. No funds from the US Army have been used for animal experiments.

The experiments performed with TIME cells are essentially the same as the results obtained with SV-HCEC cells. No significant differences were observed in dose-response experiments, responses to cytokines, and calcium and ROS inhibitors.

III. KEY RESEARCH ACOMPLISHMENTS:

Further refinement of the in vitro models of endothelial barriers (dermal and cerebral) by using human derived microvascular endothelial cells of dermal and cerebral origin.

Demonstration of the role of rickettsiae, cytokines, ROS and NO in modulation of endothelial barrier functions.

Demonstration of the partial role of intracellular calcium in modulating permeability across the endothelial monolayer.

Successful development of a protocol to isolate primary brain microvascular endothelial cells of murine origin to study tight junctional changes as they relate to changes in permeability

Successful transfection of human microvascular endothelial cell lines with adaptein-containing retroviruses (6-, 12-, and 18-mer peptides).

Rickettsial challenges are partially successful. Expansion of the resistant colonies is still elusive. Use of cell sorters in a flow cytometer and experiments with macrophages grown in suspension could help us achieve better results in selecting and expanding transfected cells resistant to rickettsial challenge.

IV. REPORTABLE OUTCOMES

1. Paul Koo, Michael E. Woods, **Juan P. Olano**. *In Vitro* Studies of Microvascular Permeability During *Rickettsia rickettsii* Infections. Accepted for presentation at the 4th International Conference on Rickettsia and Rickettsial Diseases. Logrono, Spain. 2005.
2. Michael E. Woods, Paul Koo, Gary Wen and **Juan P. Olano**. Nitric oxide (NO) as a mediator of increased microvascular permeability during rickettsial infection. Accepted for presentation at the 4th International Conference on Rickettsia and Rickettsial Diseases. Logrono, Spain. 2005.
3. Walker, D.H., Valbuena, G., **Olano, J.P.** Pathogenic mechanisms of diseases caused by *Rickettsia*. In Rickettsiology: Present and future directions. Hechemi, K.E., Avsic-Zupanc, T., Childs, J.E. and Raoult, D.A., (eds). Annals of the New York Academy of Sciences. Vol 990:1-11, 2003.
4. Koo P, **Olano JP**. *In vitro* studies of endothelial permeability using human microvascular endothelial cells infected with *Rickettsia* rickettsii (in preparation).
5. Koo P, **Olano JP**. Calcium signaling and endothelial permeability in *Rickettsia rickettsii*-infected human microvascular endothelial cells (in preparation).

V. CONCLUSIONS

In summary, several goals were accomplished during the third year of this project. The greatest progress was achieved in the study of microvascular permeability in rickettsial infections and its modulation not only by rickettsiae alone but by three major cytokines that are part of the immune response in rickettsial infections as shown in animal models. The role of calcium, NO and ROS, although not as important as cytokines, also offers possible therapeutic targets to prevent or ameliorate both cerebral and pulmonary edema during the acute phase of the various rickettsioses.

Our plans to continue with the experiments involving the use of laser confocal microscopy and live-infected cells were again delayed due to several administrative regulations in our institution. Our department was finally notified

February 26, 2005 that the Olympus LV 1000 confocal microscope has arrived to our institution. Technical personnel from Olympus will be installing the microscope within the next 2 weeks and therefore we will be re-starting such critical experiments. We certainly apologize for the delays in making significant progress in experiments involving signal transduction that required the use of laser confocal microscopy.

Challenge experiments have continued and were extended to *R. prowazekii* and *O. tsutsugamushi*. Even though the results have been discouraging, we still think that our main problem is the expansion of the resistant colonies after three rounds of rickettsial challenges. Such colonies are expressing retrovirus as evidenced by their green fluorescence. The acquisition of the flow cytometer/cell sorter for use in the BSL-3 environment will allow us to modify our selection protocol for resistant cells and most likely will allow us enrich the resistant populations further for full characterization. As mentioned in our previous progress report, Drs Watowich and Davies have already developed adaptein-containing cell lines resistant to challenge by alphaviruses and the anthrax lethal toxin.

VI. SCIENTIFIC PERSONNEL

1. David H. Walker, M.D. Principal Investigator (10% effort)
2. Juan P. Olano, M.D. Co-Principal Investigator (25% effort)
3. Paul Koo Ph.D. Post-Doctoral fellow (100%)
4. Michael Woods, B.Sc. Graduate student (100% effort).
5. Gary Wen, M.Sc. (50% effort)
6. Leoncio Vergara, M.D. (10% effort, as of June 2004).

Mr Woods and Wen were added to the grant. Mr. Woods is working on the isolation of primary endothelial cell lines and the role of several signal transducers in endothelial permeability. Mr. Wen is working on challenge experiments with Dr. Koo. Dr. Vergara is an expert on confocal microscopy who has been opitimizing protocols with cell lines for calcium experiments under BSL-1 conditions (cell loading, transfection, etc). He will be collaborating with Dr. Olano in the experiments involving live-infected cells in the BSL-3 laboratory.

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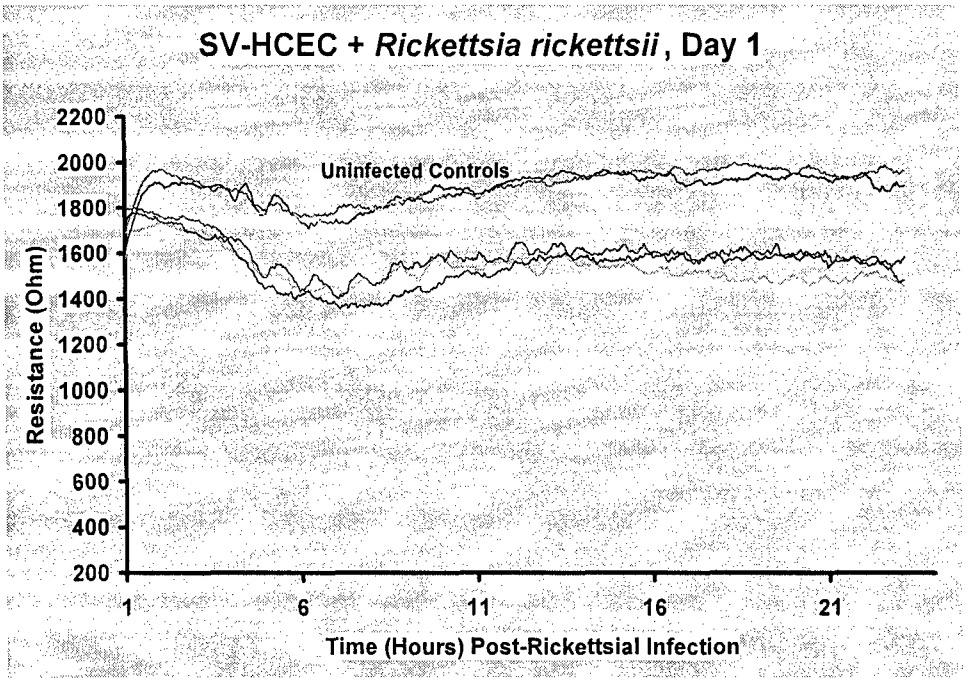
APPENDICES



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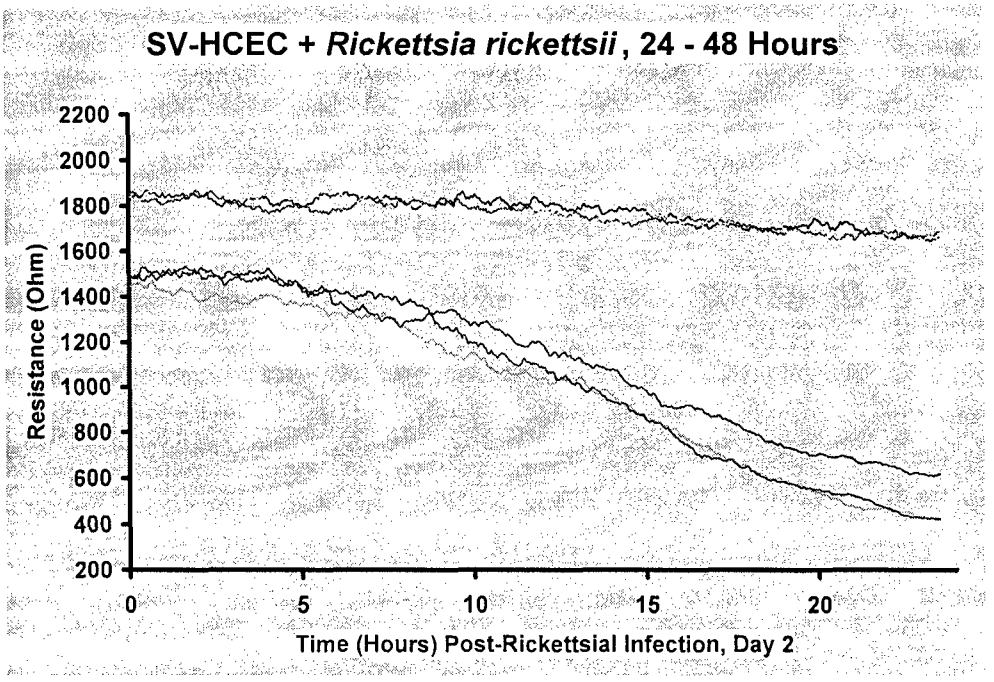

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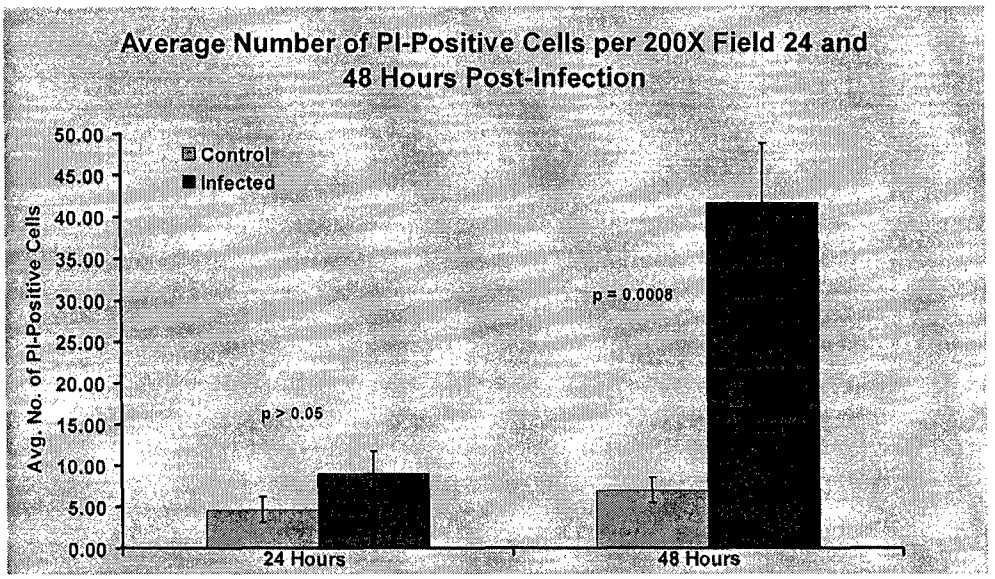

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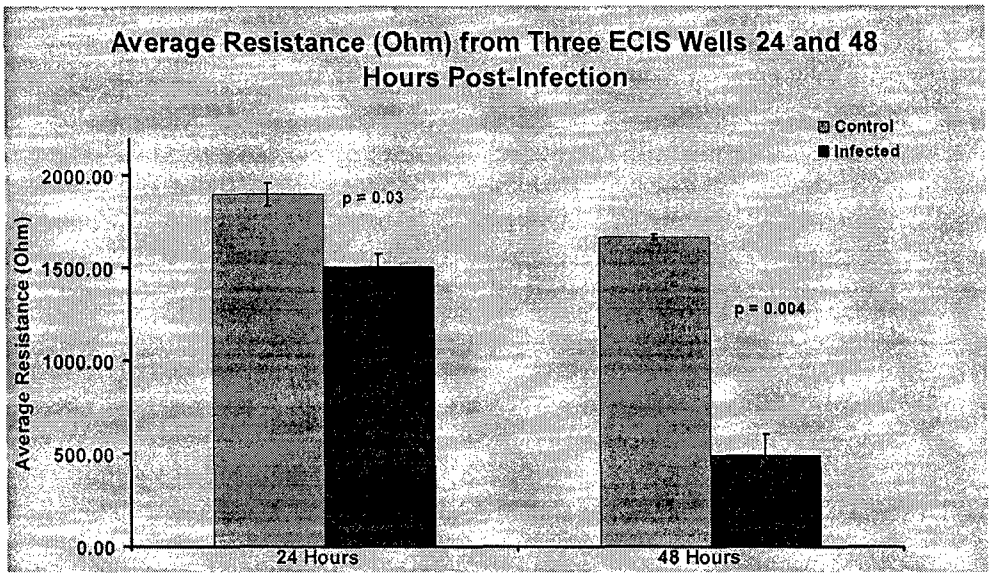

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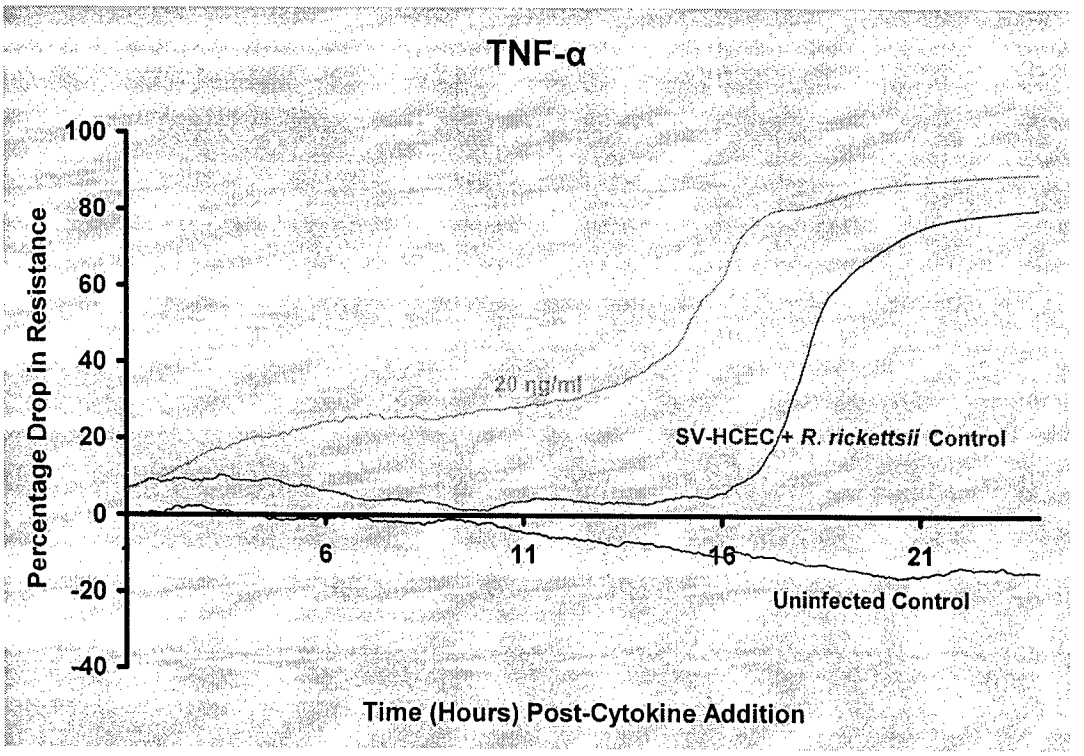

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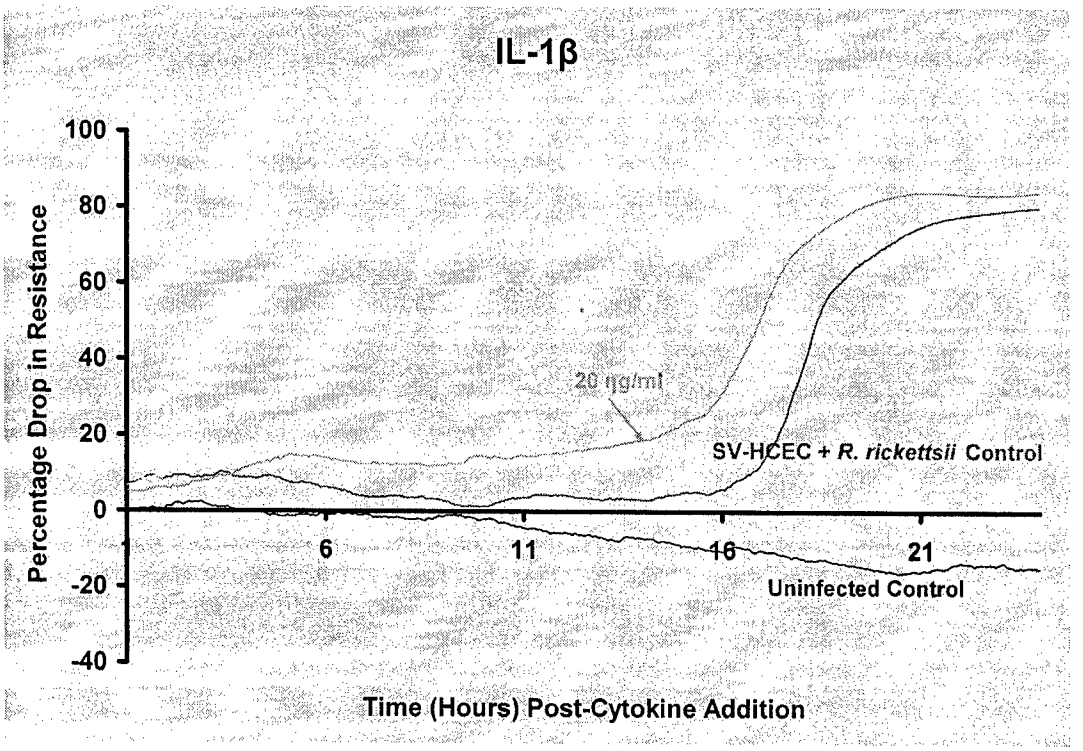

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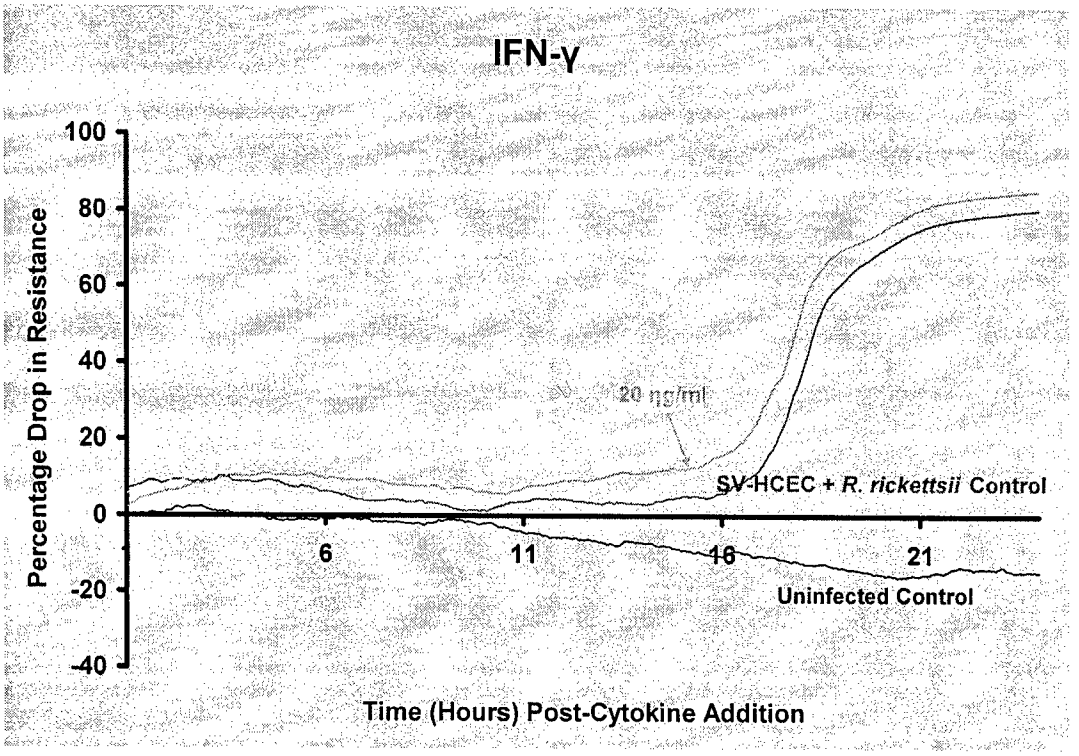

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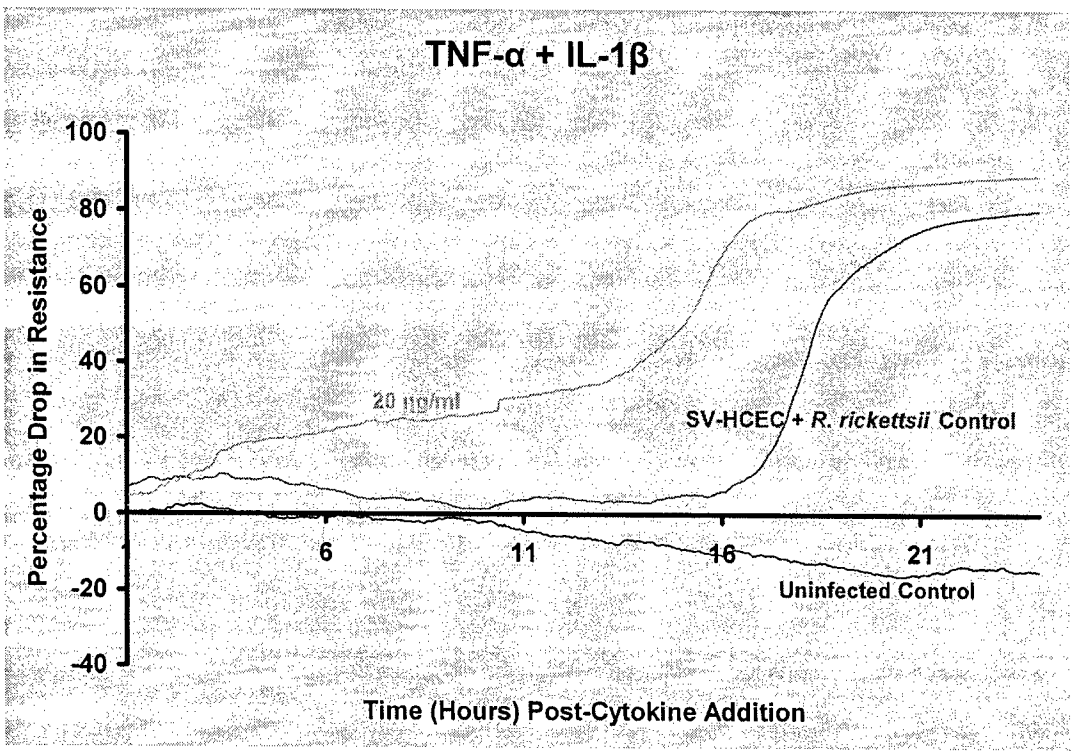

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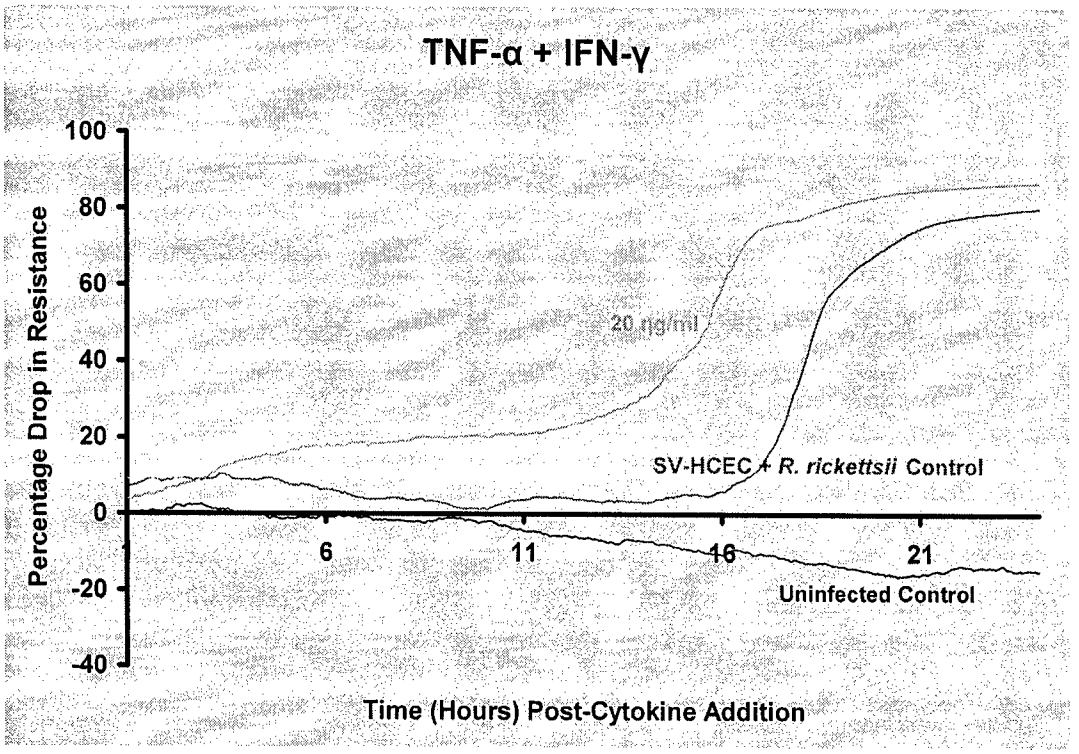

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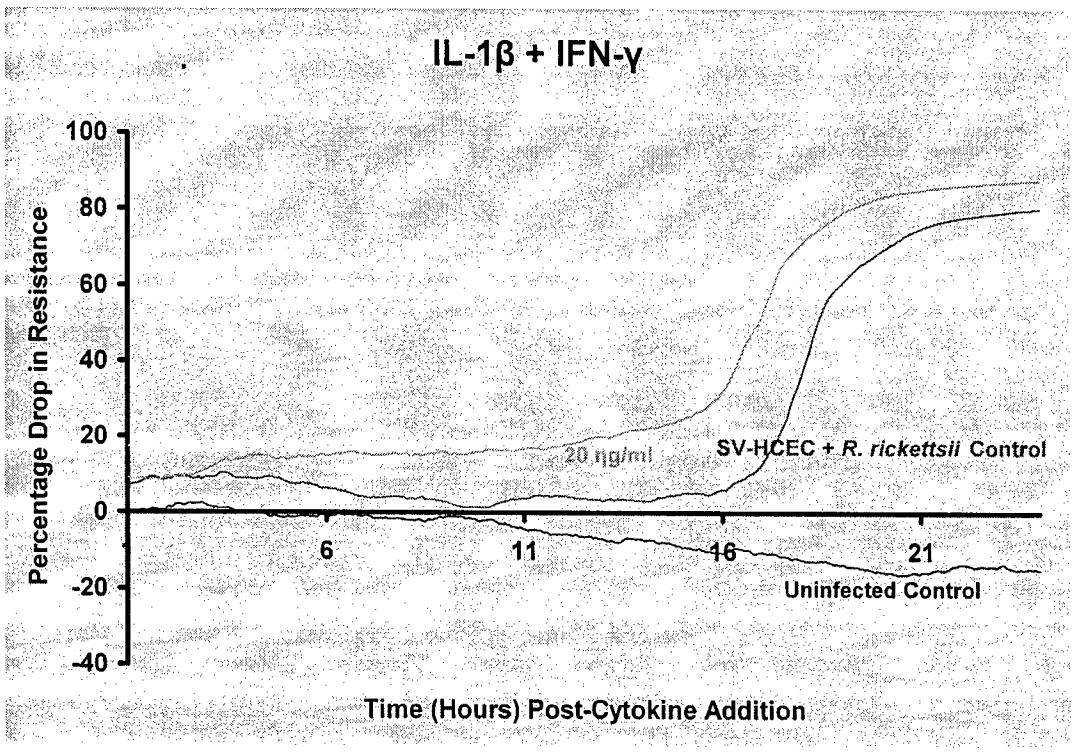

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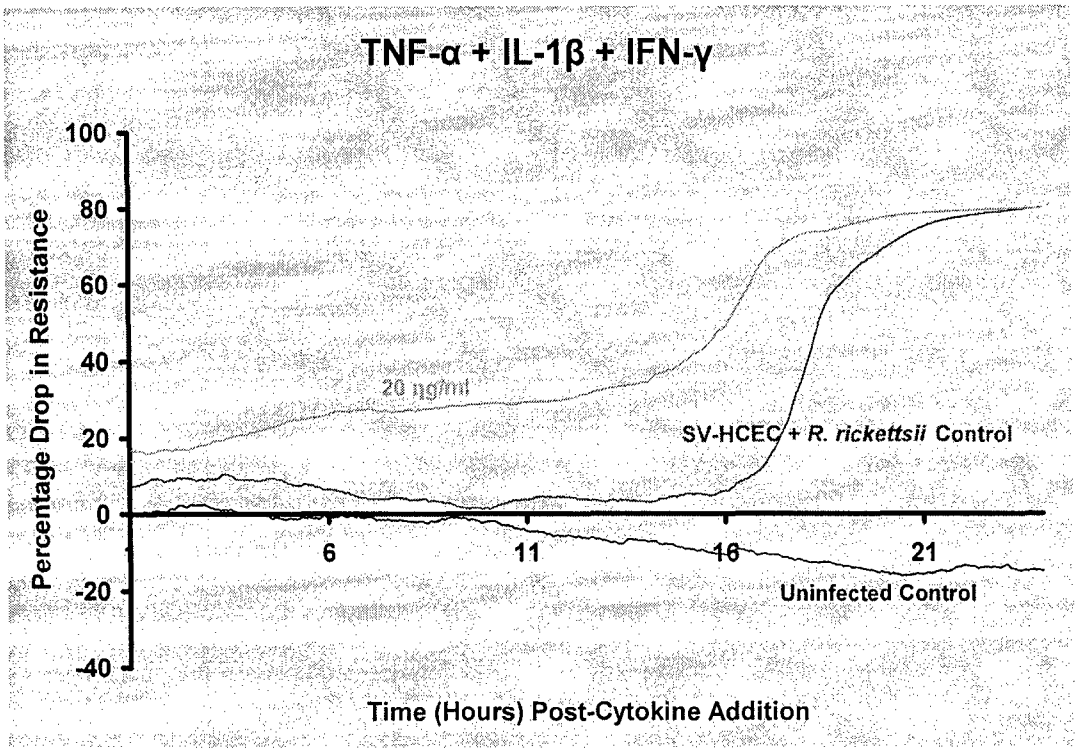
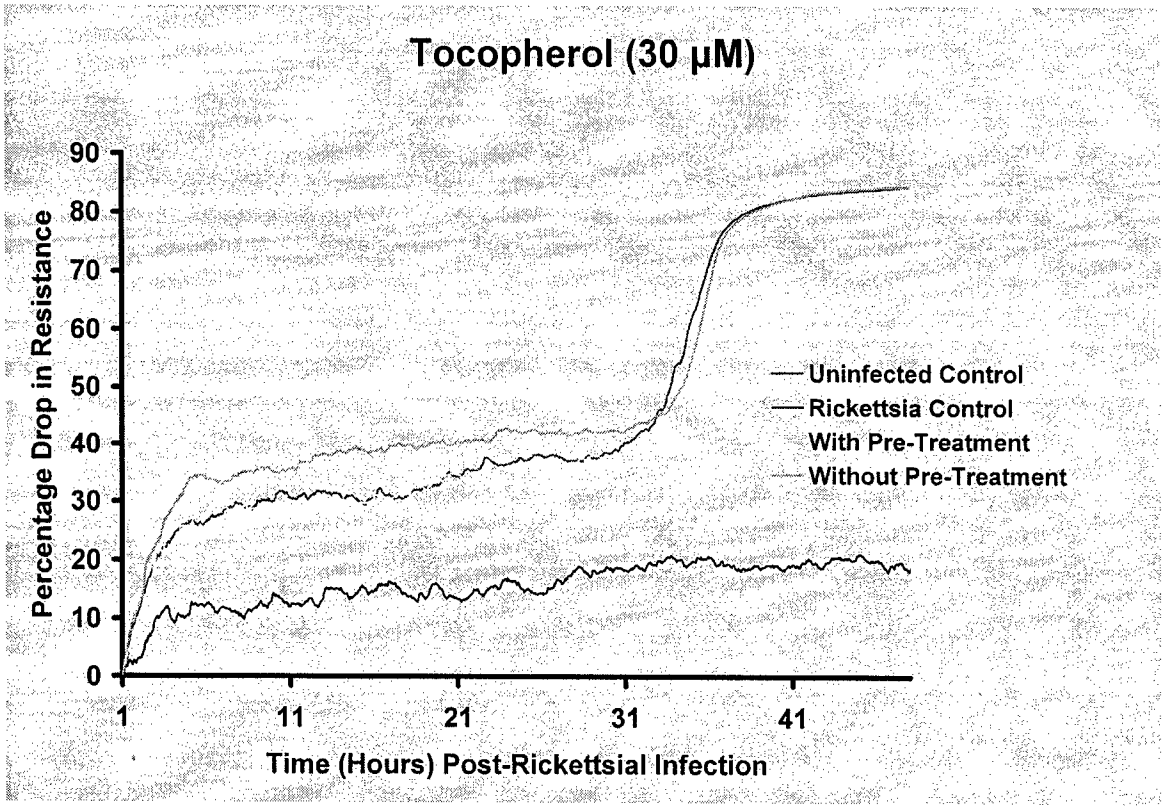

TNF-α + IL-1β + IFN-γ
Percentage Drop in Resistance
100
80
60
40
20
0
-20
-40
6
11
16
21
20 ng/ml
SV-HCEC + R. rickettsii Control
Uninfected Control
Time (Hours) Post-Cytokine Addition

Figure 9



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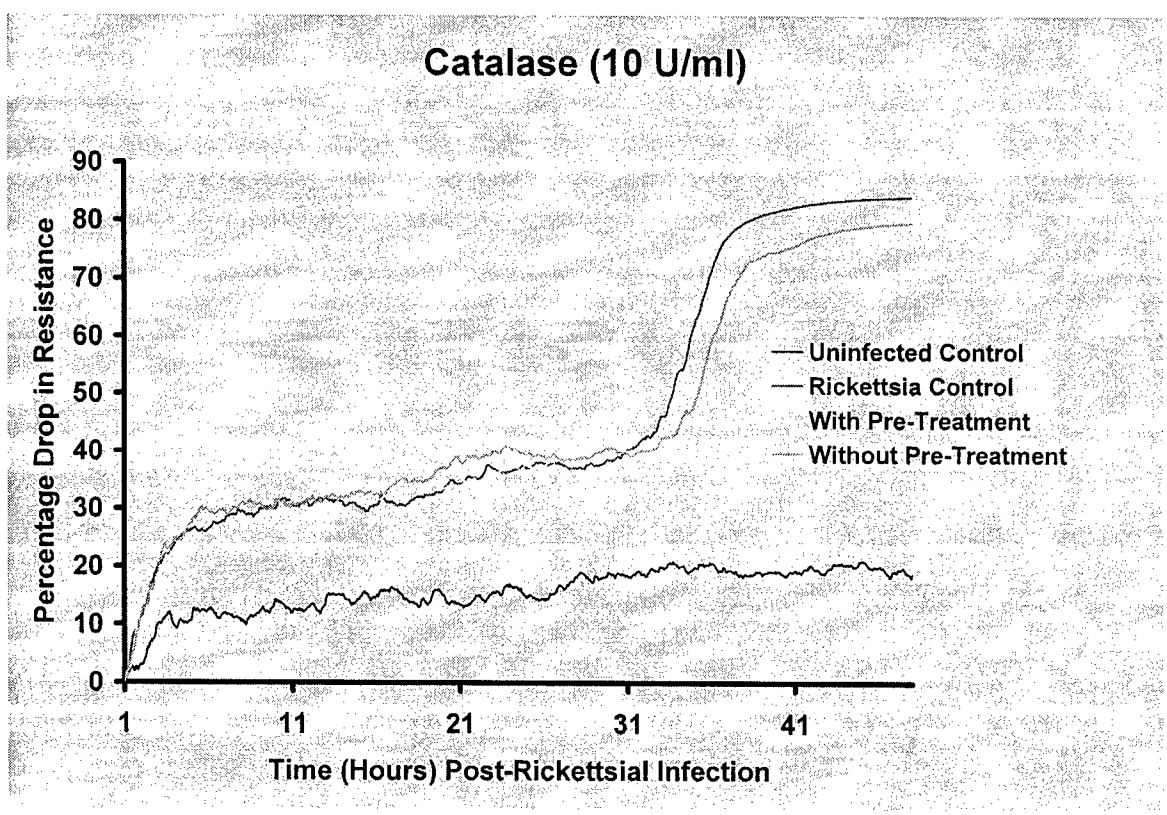
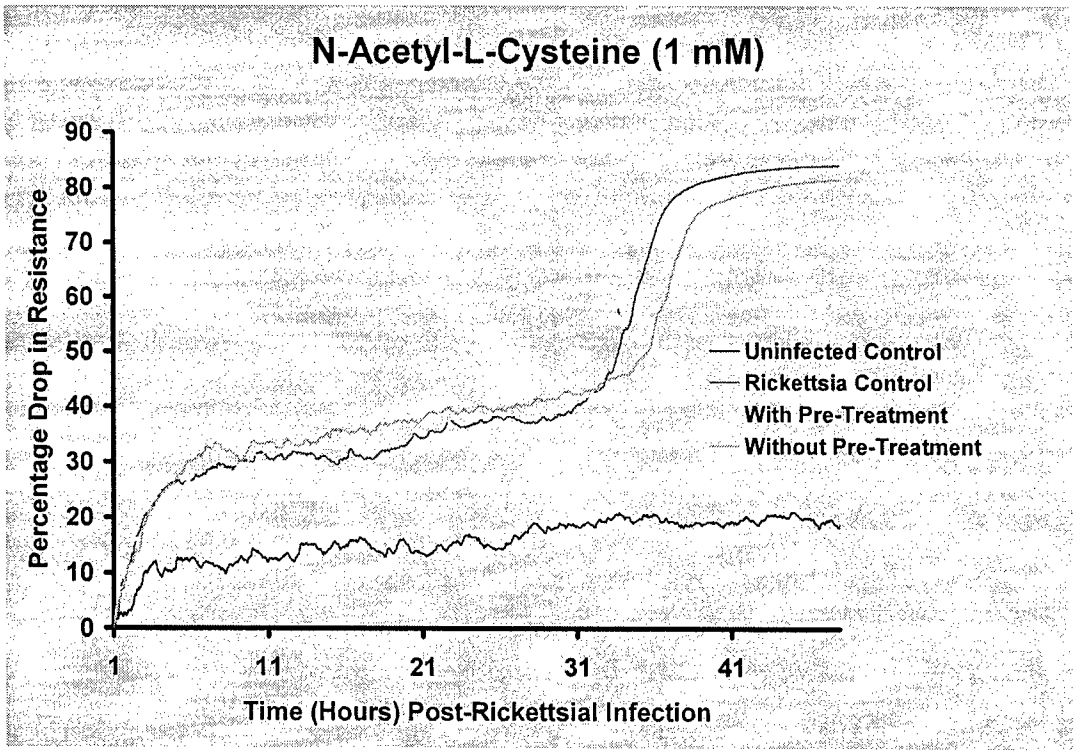


Figure 11



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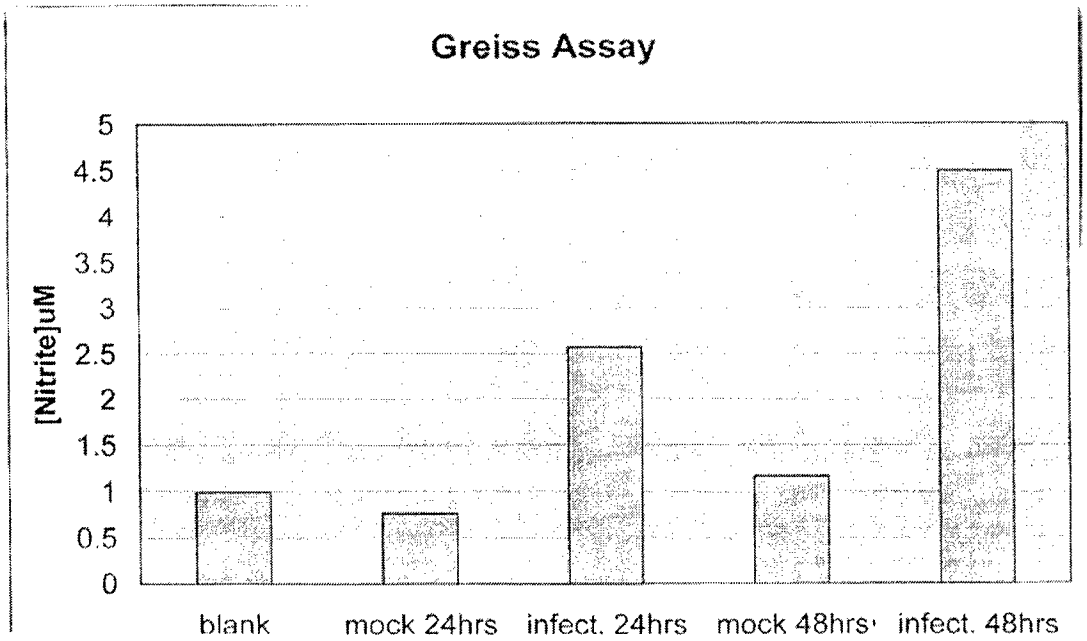

Figure 13

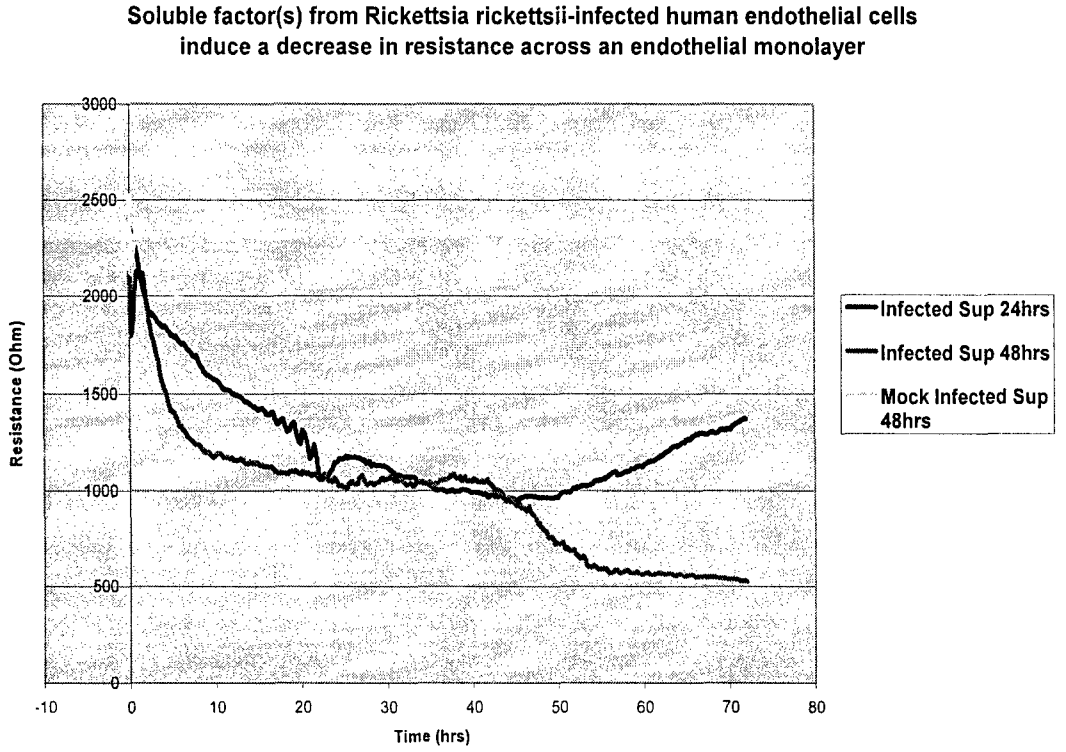

Figure 14

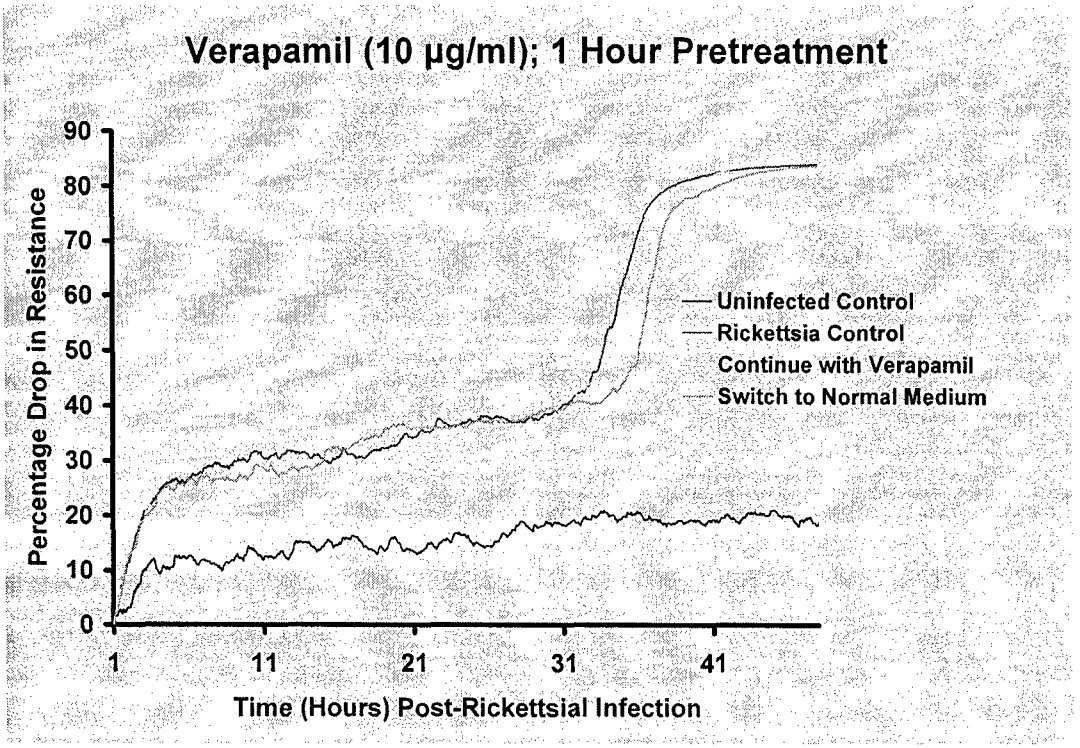

Figure 15

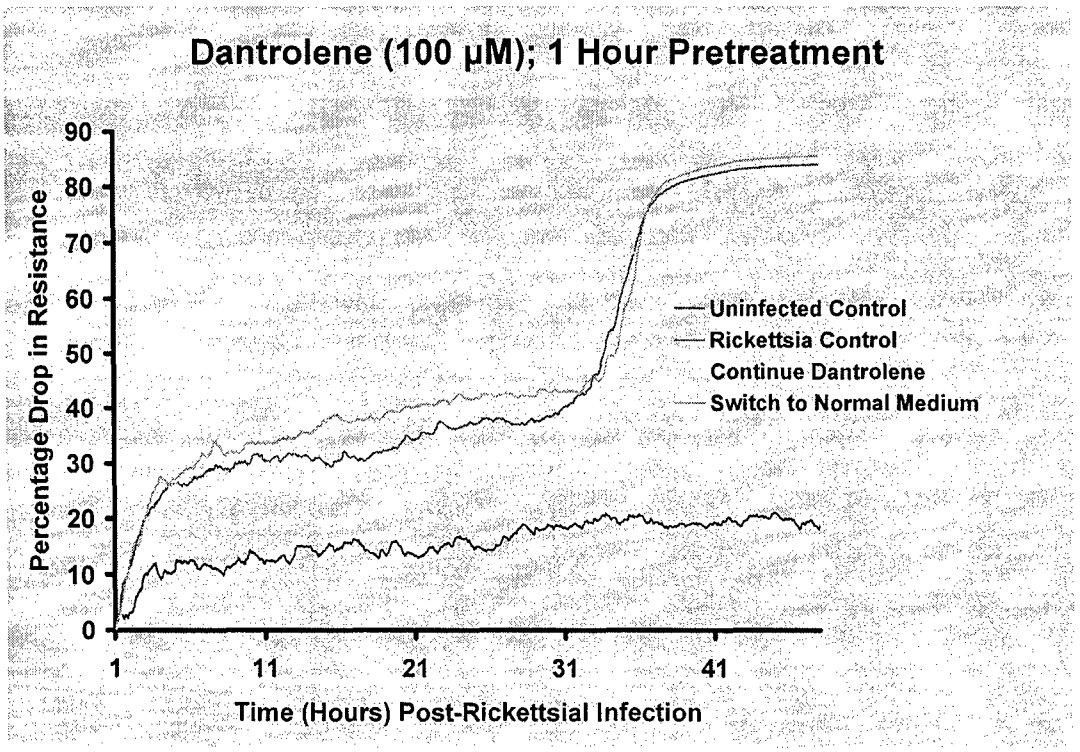

Figure 16

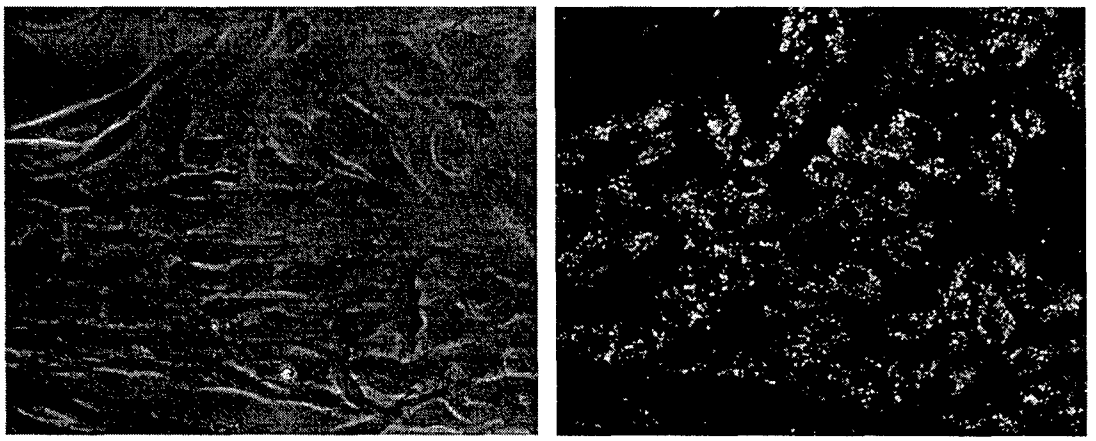

Figure 17

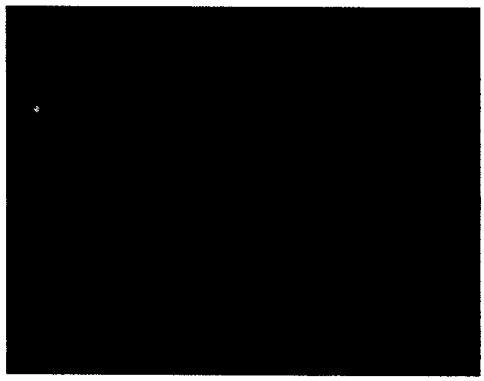

Figure 18

In Vitro Studies of Microvascular Permeability During _Rickettsia rickettsii_ Infections.

Paul Koo, Michael E. Woods, Juan P. Olano

Introduction: Acute rickettsioses are characterized by disseminated involvement of several microvascular beds throughout the body leading to the most feared complications of these diseases, namely vasogenic cerebral edema and non-cardiogenic pulmonary edema. The mechanisms responsible for increased microvascular permeability are completely unknown.

Methods: Sarcoma-virus transformed human microvascular endothelial cells (SV-HCEC) and telomerase-transfected human microvascular dermal cell lines (TIME) were used in our experiments. Permeability was evaluated by monitoring the cell monolayers by Electronic Cell Substrate Impedance Sensing (ECIS). The monolayers were infected with 10-100 MOI of renografin-purified *R. ricketsii* and permeability changes were monitored up to 72 hours after infection. Subsequent experiments were performed with 15 MOI. The effects of cytokines on endothelial permeability were evaluated by adding TNF-α, IFN-γ, and IL-1β, 24 hours after infection of the monolayers.

Results: Increases in permeability across the monolayers ranged from 20-50% at 6 hours after infection and were directly proportional to the initial inocula. Changes in permeability observed during the first 32 hours ranged from 20-25% followed by a precipitous drop at 48 hours. Changes in permeability during the first 24 hours of infection were not related to cell death. Addition of TNF-α, IL-1β and IFN-γ further increased permeability in the monolayers by 15-20%, 10-15%, and 5-10%, respectively.

Conclusion: Rickettsiae alone increased permeability in endothelial cell monolayers early after inoculation in the absence of significant cell death. This effect is greatly enhanced by the presence of cytokines, especially TNF-α and IL-1β.

Nitric oxide (NO) as a mediator of increased microvascular permeability during rickettsial infection, Michael E. Woods, Paul Koo, Gary Wen and Juan P. Olano. University of Texas Medical Branch, Galveston, Texas.

Introduction: Rickettsiae primarily target the microvascular endothelium leading to disseminated infection of the microvasculature. The mechanisms of increased microvascular permeability during rickettsioses are not well understood. The role of NO as an important anti-rickettsial agent has been well characterized in animal models. We sought to better describe the role NO plays in modulating endothelial tight junctions in human microvascular endothelial cells and how this contributes to increased microvascular permeability. **Methods:** A human microvascular dermal cell line that expresses tight junctions (HMEC-1, courtesy of Francisco Candal, CDC) were grown to confluence on 8W10E gold-coated electrodes and transendothelial electrical resistance was monitored by Electric Cell-substrate Impedance Sensing, or ECIS. The cells were infected with *Rickettsia conorii* (Malish 7) followed by the addition of the NO donors DETA NONOate and SNAP. Similarly treated cells were stained for the tight junction protein occludin and examined by laser confocal microscopy (LCF). Additionally, intracellular proliferation of rickettsia in the absence and presence of NO donors was followed by quantitative real-time PCR. **Results:** The addition of NO donors to infected endothelial cells resulted in a marked increase of transendothelial permeability as measured by ECIS. This increase occurred within the first 24 hours of stimulation. Light microscopic observation of endothelial cell morphology revealed no drastic changes in cell shape. However, preliminary staining of the tight junction-associated protein occludin appears to demonstrate disassembly of the tight junction complex by the loss of occludin staining at intercellular borders by LCM. Quantification of intracellular rickettsiae by quantitative real-time PCR is currently in progress. **Conclusion:** The addition of NO donors appears to cause a dramatic and immediate increase in transendothelial permeability in an in vitro model of rickettsial infection. Reorganization of endothelial tight junctions as determined by LCM appears to explain the observed changes in permeability. The ability of these NO oxide donors to limit intracellular proliferation of rickettsiae has not been determined at this point. Future experiments are planned using primary mouse brain microvascular endothelial cells.